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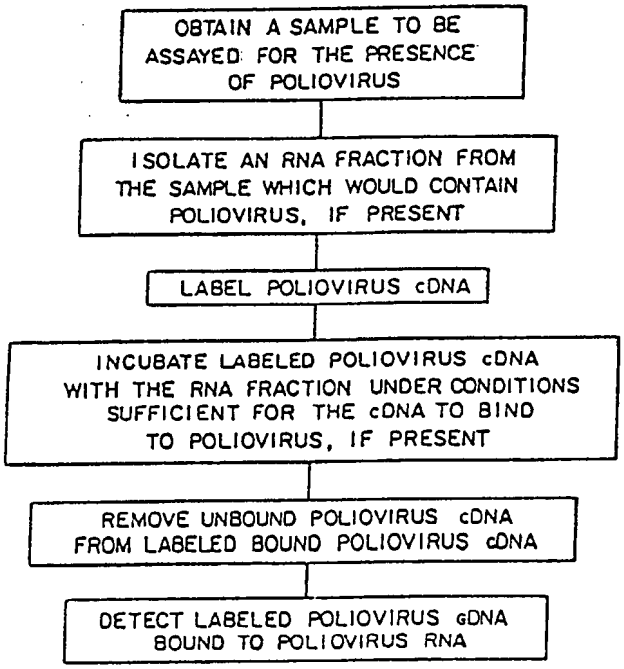
(54) Title: cDNA REPRESENTING RNA VIRAL SEQUENCES

(57) Abstract

Methods for producing RNA viral cDNA, such poliovirus ds cDNA, products thereof, and uses thereof. Poliovirus cDNA is produced, for example, by reverse transcribing poliovirus RNA and subsequently inserting the poliovirus cDNA into bacterial plasmids by genetic-engineering techniques. Transformed bacteria are then cloned and cultured to produce replicated chimeric plasmids containing the cDNA poliovirus. Such poliovirus cDNA is useful in assaying for the presence of poliovirus and in the production of antibodies against poliovirus. It has also been found that full-length poliovirus cDNA is infectious, which means it can be employed in producing altered virus particles for vaccines.



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cDNA REPRESENTING RNA VIRAL SEQUENCES

Description

Technical Field

This invention is in the field of microbiology and more specifically relates to recombinant DNA techniques for producing genetically-engineered microorganisms.

Background Art

Poliovirus, one of the human picornaviruses, has been extensively studied because it is the causative agent for serious human disease. Because of these studies, it is known that the virion of poliovirus consists of a small icosahedron, 25-30 nm in diameter, composed entirely of four polypeptides, which are designated VP1, VP2, VP3 and VP4. A single strand of infectious positive-stranded RNA of molecular weight 2.7×10^6 daltons is enclosed within this protein coat. This size is equivalent to approximately 7500 bases, which can code for about 2500 amino acids.

Despite the extensive studies made of poliovirus, there still remain many problems with the current techniques available for the study, detection and production of this virus, as well as with the techniques used to produce antibodies against poliovirus.

For example, the need for improvements in techniques for detection can be seen when it is recognized that poliovirus RNA cannot be practically employed in the detection of poliovirus. This is because poliovirus RNA is in short supply, is unstable, and does not normally bind to other poliovirus RNA.

To date, the major assay for detecting the presence of poliovirus is a biological technique in which samples are analyzed by a plaque assay employing human cell lines to detect the presence of virus. See Dulbecco, R. and Vogt, M., J. Exptl. Med. 99, 167 (1954). This procedure is relatively time consuming and expensive.

Other RNA viruses, many of which have not been as extensively studied as poliovirus, present analogous or even worse problems than poliovirus, in their study, detection, production or use in preparing vaccines or antibodies.

Disclosure of the Invention

This invention relates to the production of complementary DNA representing RNA viral sequences (RNA viral cDNA) and to methods for using such RNA viral cDNA.

In one embodiment, RNA viral cDNA is produced by reverse transcribing viral RNA and inserting the resulting cDNA molecule into a recombinant DNA vector. Appropriate cells are then transformed with the recombinant DNA vector, cloned and grown under conditions sufficient for production of RNA viral cDNA. This cDNA can then be harvested from the clonal cell culture and used, as is, or further modified for certain applications.

In a particular embodiment, bacteria are modified by genetic engineering techniques to make such bacteria capable of producing poliovirus double-stranded complementary DNA (ds cDNA). In this method, poliovirus single-stranded (ss) RNA is reverse transcribed to provide poliovirus ss cDNA which is extended to ds cDNA and then inserted into a bacterial plasmid to create a chimeric plasmid. The chimeric plasmid containing the ds cDNA is then inserted into bacterial cells by transforming the bacterial cells with the chimeric plasmid. Bacterial cells which have been so transformed can then be cloned and clonal cell lines grown in cell culture to replicate the chimeric plasmid. The poliovirus ds cDNA can then be recovered by enzymatically cleaving it from replicated chimeric plasmids.

This method provides for the microbiological production of relatively large quantities of RNA viral cDNA at reasonable costs. The cDNA, in turn, can be employed in assays for the detection of RNA viruses, such as poliovirus, since the RNA viral cDNA will bind specifically to viral RNA. Such assays can be performed quickly and easily and they offer the potential for being extremely sensitive for RNA virus detection.

RNA viral cDNA can also be employed in the production of either more RNA viral antigen or antibodies to such an antigen. In these methods, cDNA to viral RNA is produced, as described above. For antigen production, cDNA capable of stimulating antigen production is selected and inserted into cells capable of producing the antigen after which the cells are cultured under conditions suitable for antigen production, and antigen is then harvested. For antibody production, harvested antigen is

employed to immunize a host capable of producing antibodies to the original virus employed. Monoclonal antibodies can be produced employing antibody-producing cells from the host and known techniques, such as the formation of hybridoma cell lines.

Surprisingly, it has been found that a full-length poliovirus cDNA molecule produced by the methods described herein and transfected into cells is itself infectious. Such infectious cDNA molecules offer great potential in the production of viral antigens, antibodies and vaccines over their counterpart RNA molecules. For example, a cDNA molecule can be mutagenized with known recombinant DNA techniques. The mutagenized cDNA can be transfected into cultured cells and the resulting viral particles will contain the desired alteration. Such RNA viral particles may offer distinct advantages over their wild-type counterparts in the production of vaccines.

Brief Description of the Drawings

FIGURE 1 is a schematic diagram illustrating the production of a bacterial chimeric plasmid containing poliovirus ds cDNA;

FIGURE 2 is a block diagram illustrating one embodiment of an assay employing poliovirus cDNA which could be produced according to the methods described herein;

FIGURE 3 is a schematic diagram illustrating the length of separate poliovirus ds cDNA's produced according to this invention; and

FIGURE 4 is a schematic diagram illustrating the method employed to splice two poliovirus ds cDNA's (pVR104 and pVR105) to produce a full-length poliovirus ds cDNA (pVR106).

Best Mode for Carrying Out the Invention

As used herein, the terms "poliovirus RNA," "poliovirus cDNA," "picornavirus RNA," "picornavirus cDNA," "viral RNA," etc., mean the whole RNA or DNA molecule or any significant portion thereof. Thus, the term "poliovirus cDNA" is used to mean DNA complementary to the entire poliovirus RNA or DNA complementary to any significant portion of the poliovirus RNA molecule.

The methods described herein for producing viral RNA cDNA employ fundamental gene splicing techniques which have been described in the scientific literature. For example, U. S. Patent No. 4,227,224, issued to Stanley N. Cohen and Herbert W. Boyer, on December 2, 1980, describes many of these techniques. The teachings of the Cohen and Boyer patent, therefore, are incorporated herein by reference.

A more specific description of the techniques which can be employed in producing poliovirus ds cDNA will now be presented in conjunction with Figure 1, a schematic diagram illustrating these techniques.

Type 1 poliovirus is employed. Such virus can be obtained by growing epithelioid cells in suspension culture and infecting the culture with poliovirus, Type 1. The infected cells are then lysed with detergent to release virus particles, which are purified by centrifugation.

Poliovirus ss RNA can be extracted from the purified viral particles by phenol-chloroform extraction. The extracted ss RNA is then precipitated by ethanol precipitation.

The poliovirus ss RNA is then employed in the synthesis of poliovirus ds cDNA, as illustrated. Initially, the poliovirus ss RNA is reverse transcribed employing the enzyme reverse transcriptase, also known as RNA-dependent DNA polymerase. See Kacian, D. L. and Myers, J. C. (1976) PNAS 73:2191-5. Typically, Tris-HCl buffer, pH 8.3, magnesium ions (Mg^{++}), dithiothreitol, the four-deoxynucleoside triphosphates (dATP, dCTP, dGTP and TTP), and at least one labeled deoxynucleoside triphosphate for monitoring the product are added to the reaction mixture. Oligo(dT) is also added as a primer which hybridizes to the poly(A) end of poliovirus RNA thereby providing a site for initiation of reverse transcription. The reaction mixture is incubated under conditions to allow the enzyme to synthesize a complementary ss DNA copy of the poliovirus genome starting from the 3' poly(A) end and continuing to the 5' end of the genome. The reaction can be halted by the addition of ethylene diamine tetraacetic acid (EDTA).

The RNA template is then removed with alkali and the ss cDNA molecules are fractionated on a sucrose density gradient. Larger molecules are typically kept. These larger molecules are then placed in another reaction mixture containing Tris-HCl buffer, pH 7.5, Mg^{++}, dithiothreitol, the 4-deoxynucleoside triphosphates, and the Klenow fragment of DNA polymerase I. This reaction mixture is maintained under conditions sufficient to allow the DNA polymerase I to extend the cDNA molecule initiating synthesis at the snap-back formed at the 3' end of the molecule. In a typical example, the reaction mixture might be incubated at 37° for about 30 minutes, which is usually sufficient for formation of the second complementary DNA strand, as illustrated. See Humphries et al. (1978), Nucleic Acids Res., 5:905-24.

S1 nuclease is then employed to cleave the loop at one end of the molecule. See Bhat and Piatigorsky (1975), PNAS, 76:3299-3303.

The ds cDNA can then be tailed with oligo(dC) at the 3' ends by employing terminal transferase and dCTP. See Boyer et al. (1977) in "Recombinant Molecules: Impact on Science and Society" (R. F. Beers and E. G. Bassett, eds.) pp 9-20, Raven, N. Y. The tailed ds DNA can be electrophoresed on an agarose gel, and the largest fragments are then eluted from gel slices by electrophoresis. This leaves ds cDNA having poly(C) at both ends to serve as "sticky" ends in subsequent binding to a cleaved bacterial plasmid in order to form a recombinant DNA molecule.

Plasmid pBR322 can be employed to illustrate chimeric plasmid formation. Plasmid pBR322 is a well characterized plasmid known to contain selectable markers. This plasmid contains one gene coding for tetracycline resistance as well as a gene coding for ampicillin resistance. Since the poliovirus ds cDNA sequences are inserted into the gene for ampicillin resistance, successfully transformed bacterial cells are ampicillin sensitive (Amp^S) and tetracycline resistant (Tet^R), the latter providing a marker for transformed cells.

Plasmid pBR322 is cleaved using the restriction enzyme Pst I at the gene coding for ampicillin resistance. The resulting linearized plasmid is then tailed with oligo(dG) employing the enzyme terminal transferase and dGTP to produce "sticky" ends on the linearized cleaved plasmid chains. These plasmid chains can be purified by phenol extraction.

The oligo(dG) tailed plasmid DNA and the oligo(dC) tailed poliovirus ds cDNA are then hybridized in solution. This can be accomplished by mixing these DNA species in an equimolar ratio in 0.1 M NaCl, heating for 2 minutes at 68° and then incubating at 45° for 3-4 hours. See Boyer et al., 1977.

The hybridized plasmid-poliovirus ds cDNA is then inserted into E. coli in order to reconstruct the Pst I site, to amplify the plasmid DNA, and to identify clones which contain recombinant plasmids. See Dagert, M. and Ehrlich, S. D. (1979), Gene, 23-28. Once the hybrid molecule is inserted, the single-stranded gap is repaired by the bacteria. This reconstruction provides a Pst I site which the Pst I enzyme can later recognize and cleave to separate the poliovirus ds cDNA sequences from replicated plasmids.

E. coli cells transformed with the hybrid molecules can then be selected in the presence of tetracycline and later screened for ampicillin sensitivity. Those clones identified as Tet^r Amp^s can then be analyzed by colony hybridization in order to detect specific poliovirus sequences in isolated clones. See Grunstein and Hogness (1977), PNAS 72:3961-5. Tet^r Amp^s clones can be grown on nitrocellulose filters on top of agar medium. Colonies are lysed on the nitrocellulose and the DNA is fixed to the filters. The DNA on the filters is then hybridized with ^{32}P-labeled poliovirus cDNA in sealed polyethylene bags. Autoradiography of the washed and dried filters reveals which colonies contain specific poliovirus sequences, since the DNA from these colonies

will hybridize to the labeled poliovirus cDNA and appear as dark spots when the filters are exposed to x-ray film.

Plasmid DNA from positive clones can be obtained using known techniques. See, for example, Meagher et al. (1977) and Guerry et al. (1973).

The DNA can be subjected to electrophoretic analysis on agarose gels after digestion with Pst I. Comparison of the digestion pattern of pBR322 and the hybrid plasmids obtained from positive clones indicates the length of the inserted DNA.

Those skilled in the art will recognize, of course, that other materials and conditions can be employed other than those specifically described in the aforementioned embodiment. For example, although Type 1 poliovirus was employed, it is believed that either Type 2 or Type 3 could also be employed, if desired. Additionally, it is clear that bacterial cells other than E. coli could be employed. For example, B. subtilis could also be employed as well as many other bacterial strains.

As will be clear to those skilled in the art, the methods described above are not limited to poliovirus and are equally applicable with other RNA viruses. This is particularly true, of course, for RNA viruses having a genome formed from a single positive strand of RNA. These include: picornaviruses other than poliovirus, such as coxsackieviruses, rhinoviruses, and foot and mouth disease viruses; and togaviruses, such as Type A (Alphaviruses) and Type B (Flaviviruses).

Similarly, although bacterial plasmids have been employed in producing poliovirus cDNA sequences, other recombinant DNA vectors could be employed. Examples of other recombinant DNA vectors include phages, animal viruses and yeast vectors. Hosts which allow the recombinant DNA vector to multiply are chosen, of course.

One significant use for RNA viral cDNA produced according to this invention, such as poliovirus cDNA, is in assays to detect the presence of an RNA virus. In a typical assay for poliovirus, for example, a patient sample, such as cerebrospinal fluid, can be assayed as illustrated in Figure 2. The RNA fraction of the patient sample is first isolated, which can be done by phenol extraction and ethanol precipitation. This RNA fraction need not be pure, but it must be a fraction which would contain poliovirus RNA if poliovirus were present in the original sample. Poliovirus cDNA is first labeled, e.g., with a radioactive material such as tritium, iodine, or ^{32}P, and subsequently incubated with the RNA fraction under conditions to allow the labeled poliovirus cDNA to bind to poliovirus RNA, if present. After incubation, unbound labeled poliovirus cDNA is separated and bound labeled cDNA poliovirus is then detected in a scintillation counter or by other means.

Other patient samples, of course, such as blood serum or a biopsy, might be employed. Additionally, the assay can be performed on other liquid samples which might contain poliovirus, such as sewerage. Similarly, the assay can be employed for RNA viruses other than poliovirus.

A solid-phase assay, although not illustrated, might be performed. Additionally, the label need not be a radioactive isotope, but might be an enzyme, optical label, etc.

Another significant use for RNA viral cDNA produced according to this invention is in the production of antibodies against an RNA virus or viral particle.

Antibodies could be produced by reverse transcribing viral RNA to provide cDNA, inserting the cDNA into a recombinant DNA vector and transforming cells in which said recombinant DNA vector can multiply. Transformed cells can then be cloned to produce a cell line capable of replicating the cDNA, the cell line can be cultured under conditions sufficient for the production of cDNA and cDNA can then be harvested from the cell culture. Specific cDNA could be selected and isolated which was capable of directing antigen synthesis in cells and subsequently inserted into cells so that these cells would produce antigen. A host, such as an animal, could then be immunized with the antigen to cause the host to produce antibodies against the original RNA virus or a portion thereof.

Experiments have shown that RNA viral cDNA can be infectious, a surprising finding. These experiments were performed employing full-length, cloned cDNA copies of the RNA genome of poliovirus constructed in the Pst I site of the bacterial plasmid pBR322 (See Examples below). Cultured mammalian cells transfected with these hybrid plasmids produced infectious poliovirus. Cells transfected with a different poliovirus cDNA clone, which lacked the first 115 base pairs of the poliovirus genome, did not produce virus.

This finding means that it is possible to produce RNA virus particles by transfecting cells with cDNA for the RNA virus, culturing the cells under conditions suitable for virus production, and subsequently harvesting the RNA virus particles.

It also means that it will be possible to perform genetic manipulations not possible with RNA which may

open up a variety of new approaches to the study of RNA viruses and vaccine production.

For example, a vaccine having properties different from one produced from current vaccine strains could be prepared from cDNA as follows. Initially, a cDNA copy of the RNA genome could be produced as described herein, and the cDNA could be mutagenized employing recombinant DNA techniques. Cells capable of producing RNA viruses could then be transfected with the cDNA and cultured under conditions sufficient to produce the altered virus, which could then be employed in vaccine production.

In one specific approach, specific areas of the viral genome can be deleted from the cDNA using recombinant DNA techniques. Plasmids containing the altered cDNA could then be transfected into cells, such as mammalian cells. Virus particles produced by the cells would be recovered and assayed for attenuation in a suitable host.

Transfection into cells can be achieved by known techniques. For example, the calcium phosphate DNA co-precipitate technique, originally described by Graham and Van der Eb, is suitable. See Virology 52, 456 (1973). Similarly, the DNA/DEAE-dextran method, originally described by McCutcheon and Pagano, is also suitable. See J. Nat'l Cancer Inst. 41, 351 (1968). Those skilled in the art may know, or will be able to find using no more than routine experimentation, other suitable transfection techniques.

The invention is further and more specifically illustrated by the following examples.

EXAMPLE 1

Preparation of Hybridized Plasmid-Poliovirus ds cDNA and Cloning in E. Coli

1. Preparation of Poliovirus RNA

Poliovirus RNA was obtained using standard procedures (Flanegan et al., 1977, PNAS 74:961). HeLa cells were grown in suspension culture to a density of 4×10^5 cells per ml, centrifuged, and infected with a stock of poliovirus type 1 at a multiplicity of infection (MOI) of 10. Infection was allowed to proceed for 6 hours at 37°, at which time the cells were centrifuged. Virus was released from cells with detergent and purified from the cytoplasm by cesium chloride equilibrium centrifugation. RNA was then extracted from virions by phenol-chloroform extraction and ethanol precipitation. (See Flanegan et al., 1977.)

2. Synthesis of Poliovirus Double-Stranded cDNA

a. First Strand Synthesis

Poliovirus cDNA was synthesized in a 0.5 ml reaction mixture containing poliovirus RNA (50 µg/ml), 50 mM Tris HCl (pH 8.3), 10 mM $MgCl_2$, 50 mM KCl, 0.4 mM dithiothreitol, 30 µg/ml oligo(deoxythymidylate), 4mM sodium pyrophosphate, 0.5 mM each of dATP, dCTP, dGTP, and dTTP, 100 µCi/ml α-^{32}P-dCTP and 150 U reverse transcriptase. The mixture was incubated

at 42° for 60' and terminated by addition of EDTA. The reaction mixture was phenol extracted and ethanol precipitated, and the pellet was resuspended in 0.2 ml of a buffer containing 0.3 N NaOH, 0.7 M NaCl, 5 mM EDTA. The reaction product was subjected to alkaline sucrose gradient sedimentation at 35,000 rpm in a Beckman SW41 rotor at 20°. Fractions of 0.2 ml were collected from the gradient and analyzed by alkaline agarose gel electrophoresis. Those fractions which contained full-length poliovirus cDNA were pooled, ethanol precipitated and resuspended in 0.05 ml of water.

b. Second Strand Synthesis

Full length poliovirus cDNA (approximately 0.5 µg) was incubated in a reaction mixture containing 10 mM Tris HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM dithiothreitol, and the Klenow fragment of DNA polymerase I (.003 U/ng cDNA). The mixture was incubated at 37° for 30' and terminated by phenol extraction. The aqueous phase was applied to a 1 x 10 cm column of Sephadex G-100 equilibrated with 0.1M NaCl, 10 mM Tris HCl (pH 7.5), 1 mM EDTA and developed with the same buffer. The void fractions of the column were pooled.

c. S_1 Nuclease Treatment

Double-stranded poliovirus cDNA, when prepared as described above, was in a volume of 1.0-1.5 ml. This material was combined with a buffer so that the final concentrations were 0.3 M NaCl, 30 mM NaOAc (pH 4.5), 3 mM $ZnCl_2$, and 5% glycerin. Nuclease S_1 was then added to the mixture in an amount previously determined to

cleave the loop at one end of the cDNA duplex without nicking the rest of the molecule. The mixture was incubated at 37° for 60 min. The reaction was stopped by phenol extraction, and the aqueous phase was dialyzed against a buffer containing 5 mM Tris HCl (pH 7.5), 0.5 mM EDTA. After dialysis, the cDNA was twice precipitated with ethanol and resuspended in 0.2 ml of water.

d. Addition of Poly(deoxycytidylate) to the Poliovirus ds cDNA

The cDNA from the previous step [0.05-1µg] was added to a 0.1 ml reaction mixture containing 0.15 M cacodylic acid, 1 mM $CoCl_2$, 0.2 mM dithiothreitol, 50 µg/ml bovine serum albumin and 0.15 mM dCTP. An excess of terminal deoxynucleotidyl transferase [6 U] was added and the reaction was allowed to proceed at room temperature for 20'. At the end of this time, the reaction mixture was phenol extracted, ether extracted twice, and ethanol precipitated. The final pellet was resuspended in 0.05 ml of 0.1 M NaCl, 10 mM Tris HCl (pH 7.5), 1 mM EDTA. The material is referred to as C-tailed poliovirus ds cDNA.

3. Molecular Recombination of Poliovirus ds cDNA

a. Preparation of Vector

DNA of the plasmid pBR322 was cleaved with restriction endonuclease Pst I in a 0.1 ml reaction mixture containing DNA [500 ug/ml], 15 mM Tris.HCl (pH 7.5), 5 mM $MgCl_2$, 50 mM NaCl and excess enzyme. The reaction was incubated at 37° for one hour and stopped by phenol extraction. The plasmid DNA was ethanol precipitated and resuspended in 0.02 ml of water.

Poly(deoxyguanylate) was added to the 3' ends of the plasmid DNA by the procedure described in section 2(d), above. The differences were: (1) dGTP was used instead of dCTP; (2) the reaction volume was increased to 0.3 ml, (3) the reaction was allowed to proceed at 20° for 30-60 seconds. After the reaction, the mixture was phenol extracted and chromatographed on a 1 cm x 10 cm column of Sephadex G-100 in 10 mM NaCl, 1 mM Tris-HCl(pH 7.4), 0.1 mM EDTA. The void fractions were pooled, and concentrated 10-fold under a stream of nitrogen gas. This material is referred to as "G-tailed pBR322 DNA."

b. Annealing of Poliovirus cDNA and Vector Plasmid

An equimolar amount of G-tailed pBR322 DNA and C-tailed poliovirus ds cDNA was mixed in a buffer containing 1 µg/ml G-tailed pBR322 DNA, the appropriate amount of poliovirus cDNA, 0.1 M NaCl, 10 mM Tris.HCl(pH 7.5), 1 mM EDTA. The mixture was heated to 68° for 2' and then placed at 45° for 3-4 hours. After this period, the annealed material was stored at 4° until transformation was performed.

c. Transformation

Cells of the bacterium E. coli were made competent for transformation by the following procedure. Cells were grown in 100 ml of L-broth to an optical density at 550 nanometers of 0.1. The cells were centrifuged, resuspended in 20 ml of cold 0.1 M $CaCl_2$ and placed on ice for 25 minutes. After this time, the cells were centrifuged, resuspended in 1.0 ml of cold 0.1 M $CaCl_2$, and held on ice for 24 hours.

Annealed DNA from 3(b) above [0.001 µg] was added to 0.1 ml of competent E. coli and incubated on ice for 15'. This mixture was then transferred to 37° for 5 minutes; then 1 ml of L-broth was added and the mixture was shaken for 1 hour at 37°. After this period, 3 ml of soft agar were added and the mixture was poured onto a plate of L-agar containing 15 µg/ml tetracycline. Plates were incubated at 37° until bacterial colonies were visible (usually 18 hours).

d. Identification of Poliovirus-Specific Clones

Bacterial colonies on tetracycline plates were transferred by toothpick to an array on one L-agar plate containing 50 µg/ml ampicillin, and another tetracycline-containing agar plate. Colonies which were identified as ampicillin-sensitive, tetracycline resistant were removed by toothpick to an array on a new tetracycline plate, and allowed to grow 18 hours at 37°. These colonies were then screened for poliovirus DNA using the colony hybridization technique of Grunstein and Hogness, (1975), PNAS 72:3961-5. Briefly, colonies were transferred to nitrocellulose filters, the bacteria were lysed on the filter and the bacterial DNA was fixed onto the nitrocellulose.

The filters were then hybridized to an isotopically labeled poliovirus cDNA probe, washed, and autoradiographed. Colonies which retained the radioactive probes were identified as containing poliovirus cDNA sequences.

Many positive clones were examined by isolating plasmid DNA, cleaving with restriction endonuclease Pst I, and determining the size of the poliovirus DNA insert by electrophoresis on agarose gels. Those plasmids containing the longest inserts [4.0 Kbp-6.5 Kbp] were aligned on the viral genome using nucleotode sequence analysis and restriction enzyme mapping techniques.

EXAMPLE 2

Construction Of Nearly Full-Length Poliovirus cDNA Clone

The methods outlined in Example 1 were employed to generate two poliovirus cDNA's of 4.0 Kbp and 6.5 Kbp insert length. A diagram of these DNA's is shown in Figure 3, wherein they are designated pVR102 and pVR103. A full poliovirus RNA chain is also illustrated for purposes of comparison

DNA of both plasmids pVR102 and pVR103 was cleaved by incubation at 37° in a 0.1 ml reaction mixture containing 15 mM Tris HCl (pH 7.4), 5 mM $MgCl_2$, 50 mM NaCl, DNA [500 ug/ml] and a mixture of restriction endonucleases EcoRI and Bgl II. After 60 minutes, the mixture was phenol extracted, ethanol precipitated and electrophoresed on a 1% agarose gel. The largest fragments of both clones resulting from this digestion were eluted from the gel and resuspended in 0.01 ml of H_2O. 0.002 ml

of each of these DNA's was then added to a mixture containing 50 mM Tris HCl (pH 7.5), 10 mM $MgCl_2$, 1.0 mM ATP and 300 units of phage T4 DNA ligase. The reaction mixture was incubated at 15° for 16 hours. After this time, 0.002 ml of the mixture was used to transform E. coli bacteria as described above. Resulting tetracycline-resistant colonies were examined by isolating plasmid DNA as previously described. Cleavage of the plasmid DNA's with various restriction enzymes and analysis of the digests on agarose gels indicated that a near-full length poliovirus cDNA clone had been constructed. This clone (pVR104), which is illustrated in Figure 3, begins at the 3' poly(A) sequence of poliovirus RNA, contains all internal sequences and ends 115 bases from the 5' end of the viral RNA.

EXAMPLE 3

Construction of Plasmid pVR105

1. Isolation of Primer

To construct a plasmid representing the 5' end of poliovirus RNA, the technique of primer extension was employed. Plasmid pVR103 was used to isolate a primer in the following way. Plasmid DNA (100 µg of pVR103) was digested with restriction endonucleases Bam HI and Bgl II. The digestion products were separated by polyacrylamide gel electrophoresis and the slowest migrating fragment was extracted from the gel. This DNA fragment, which consisted of bases 375-3607 of pBR322 linked, at the Pst I site, to DNA representing bases 116-220 of the poliovirus genome, was treated with calf alkaline phosphatase to remove 5'-terminal phosphates. The DNA was then phosphorylated at its 5' ends with ^{32}P using γ-^{32}P-ATP and

polynucleotide kinase. The phosphorylated fragment was then cleaved with restriction endonuclease RsaI, and the cleavage products were separated by polyacrylamide gel electrophoresis. A 74-base fragment from the Bam HI site (position 220) to the Rsa I site (position 149) was purified from the gel. This fragment contained ^{32}P at the Bam HI site only, and served as the end-labeled primer.

2. Primer Extension

The labeled primer was hybridized with 2 µg of purified poliovirus RNA in the following way. The primer and viral RNA were combined in a total volume of 0.005 ml, boiled for 2 minutes, and quenched in dry ice. The mixture was then adjusted to 0.01 M Pipes.HCl pH 6.4, 0.4 M NaCl, 2 mM EDTA, 80% formamide in a total volume of 0.05 ml. This mixture was held at 42° for 4 hours and then diluted to 0.2 ml with water and precipitated with three volumes of ethanol. The mixture was reprecipitated with ethanol three additional times to remove residual formamide.

The final ethanol pellet was resuspended in a 0.05 ml reaction mixture containing 50 mM Tris.HCl pH8.3, 50 mM KCl, 0.5 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 40 µg/ml actinomycin D, and 0.5 mM each of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate. Reverse transcriptase (RNA-dependent DNA polymerase) was added, and the mixture was incubated at 42° for 60'. After this time, the mixture was made 0.3N NaOH, incubated 37° for three hours, neutralized and the reaction contents were precipitated with ethanol. The reaction products were separated on an 8% polyacrylamide gel containing 6M urea. An autoradiograph of the gel revealed that the 74-bp

primer had been extended to 220 bases in length, indicating that the reverse transcriptase had most likely extended to the first base of the viral RNA. Nucleotide sequence analysis of the primer-extended material confirmed that the extended product reached the very 5' end of the viral RNA.

3. Molecular Cloning of Primer Extended Material

The primer-extended band 220 bases in length was excised and purified from the 8% polyacrylamide gel (see above). The fragment was ethanol precipitated and suspended in a 0.1 ml mixture containing 1 mM $CoCl_2$, 0.14 M cacodylic acid, 0.2 mM DTT, 0.15 mM dCTP, 0.3 mg/ml bovine serum albumin and the enzyme terminal deoxynucleotidyl transferase. After incubation at room temperature for 20', the reaction mixture was extracted with phenol and ethanol precipitated. This treatment added a stretch of d(C) residues to the 3' end of the primer-extended fragment.

The oligo d(C)-tailed fragment was then made double-stranded using a primer of oligo $d(G)_{12-18}$ and DNA polymerase I (Klenow). The fragment was incubated in a 0.1 ml mixture containing 0.1 M Tris.HCl pH 7.5, 0.2 M $MgCl_2$, 0.1 M DTT, 1 mM each dCTP, dATP, dTTP, dGTP, 20 µg/ml oligo d(G) and the Klenow fragment of DNA polymerase I. After incubation at 37° for 60', the reaction mixture was extracted with phenol and applied to a 0.5 cm x 5 cm column of Sephadex G-100 equilibrated and developed with 0.1 M NaCl, 10 mM Tris.Cl pH 7.5, 1 mM EDTA. The void fractions were pooled and ethanol precipitated. The resulting material was subjected to a terminal transferase reaction (see above) to add stretches of oligo d(C) to its 3' ends. These tailed molecules were then cloned into the Pst I site of plasmid pBR322 using techniques described in Example 1. By using tails of

oligo d(C) to clone this fragment, the Bam HI site, which had been phosphorylated for primer extension, was restored. Tetracycline-resistant colonies obtained from transformation of this material into E. coli were therefore screened for cleavage with Bam HI. Several molecular clones were isolated which contained inserts approximately 220 bases long which were excisable with Pst I plus Bam HI. Nucleotide sequence analysis of one such clone, called pVR105, indicated that it contained DNA representing bases 1-220 of the viral RNA. Plasmid pVR105 is illustrated in Figure 3.

EXAMPLE 4

Construction of pVR106 and pVR106a, Full-Length Poliovirus ds cDNA Plasmids

The procedure employed for joining plasmids pVR104 and pVR105 to form a full-length copy of poliovirus ds cDNA in pBR322 in shown in Figure 4.

Conditions for partial Bam HI cleavage of plasmid pVR104 were determined by incubating the plasmid with enzyme at 37° for increasing periods of time. Digests were analyzed by electrophoresis in 0.6% agarose gels and examined for the presence of linear full-length molecules. The incubation time which gave reasonable yield of linear full-length molecules (which, by definition, are a permutation of molecules cut once by Bam HI) was used to digest 100 µg of pVR104. The digestion products were electrophoresed on an 0.6% agarose gel and the linear, full-length molecules were excised and recovered from the gel. These molecules were then cleaved with EcoRI and the cleavage products were separated by 0.6% agarose gel electrophoresis. An 8-Kb DNA fragment from base 220 of the viral genome through the 3' end of the genome and

extending to the pBR322 EcoRI site was identified by its size and extracted from the agarose gel.

Similarly, plasmid pVR105 was digested under conditions which yielded linear, full-length molecules, as judged by 0.6% agarose gel electrophoresis. Linear molecules were purified by gel electrophoresis and cleaved with EcoRI. The cleavage products were separated by 0.6% agarose gel electrophoresis. The fragment consisting of nucleotides 1-3607 of pBR322 united to DNA representing bases 1-220 of the poliovirus genome was identified by its size and purified.

Approximately 0.1 µg of the DNA fragments isolated from pVR104 and pVR105 (see above) were mixed and incubated in a reaction mixture containing 50 mM Tris.HCl pH 7.8, 10 mM $NgCl_2$, 20 mM DTT, 1 mM ATP. T4 DNA ligase was added and the mixture was incubated 18 hours at 15° C. After this time, the ligated DNA's were transformed into E. coli C600 as described previously. Tetracycline-resistant colonies were examined for the presence of a full-length poliovirus cDNA clone by cleavage of plasmid DNA with various restriction endonucleases. For example, a full-length clone cleaved with Kpn I and examined by 0.6 % agarose gel electrophoresis would be expected to generate fragments 8200, 2998 and 596 bases in length. Two clones were identified, pVR106 and pVR106a, which, when digested with enzymes Bam HI, KpnI, PstI, BglI, BglII and XbaI, yielded patterns consistent with those expected from a full-length clone. Nucleotide sequence analysis of the 5' end of the insert from pVR106 and pVR106a proved that the 5' end of the viral RNA was present in these molecular clones. Therefore, pVR106 and pVR106a contained a full-length cDNA copy of the poliovirus genome at the PstI site of pBR322.

A deposit of a bacterial cell line containing plasmid pVR106 has been made at the American Type Culture Collection and is identified by ATCC Accession No. 31844. This deposit consists of plasmid pVR106 in the bacterial host E. coli HB101. The plasmid was inserted into this bacterial strain, which is rec A^-, to maintain its stability. Transformation was achieved employing the procedures described in Example 1, Section 3c.

EXAMPLE 5

Sequencing of Poliovirus cDNA

The complete nucleotide sequence of the poliovirus cDNA specific insert in clone pVR106 was obtained using published techniques. See Maxam, A. M. and Gilbert, W., (1980), in Methods in Enzymology, Grossman, L. and Moldave, K., eds., Vol. 65, pp 499-559, Academic Press, New York. This sequence in shown in Table I.

In the 5' untranslated region, potential termination codons are marked by asterisks and the phase in which they occur is indicated. ATG codons in the untranslated region are underlined, and the phase in which they occur is shown. The sequence is translated into amino acids starting at base 743 through base 7339. The coding region and amino acid sequence of VPg, the protein linked to the 5' end of the viral RNA, are indicated by underlining. The location of VPg is based on the published sequence. See Larsen, G. R., Semler, B. L. and Wimmer, E., J. Virol. 37, 328-335 (1981). The positions of the virion proteins are indicated on the basis of amino acid sequence data.

TABLE 1

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         10         20         30         40         50         60         70         80         90
TTAAAACAGC TCTGGGGTTG TACCCACCCC AGAGGCCCAC GTGGCGGCTA GTACTCCGGT ATTGCGGTAC CCTTGTACGC CTGTTTTATA

        100        110        120        130        140        150        160        170        180
CTCCCTTCCC GTAACTTAGA CGCACAAAAC CAAGTTCAAT AGAAGGGGGT ACAAACCAGT ACCACCACGA ACAAGCACTT CTGTTTCCCC

        190        200        210        220        230        240        250        260        270
GGTGATGTCG TATAGACTGC TTGCGTGGTT GAAAGCGACG GATCCGTTAT CCGCTTATGT ACTTCGAGAA GCCCAGTACC ACCTCGGAAT

        280        290        300        310        320        330        340        350        360
CTTCGATGCG TTGCGCTCAG CACTCAACCC CAGAGTGTAG CTTAGGCTGA TGAGTCTGGA CATCCCTCAC CGGTGACGGT GGTCCAGGCT

        370        380        390        400        410        420        430        440        450
GCGTTGGCGG CCTACCTATG GCTAACGCCA TGGGACGCTA GTTGTGAACA AGGTGTGAAG AGCCTATTGA GCTACATAAG AATCCTCCGG

        460        470        480        490        500        510        520        530        540
CCCCTGAATG CGGCTAATCC CAACCTCGGA GCAGGTGGTC ACAAACCAGT GATTGGCCTG TCGTAACGCG CAAGTCCGTG GCGGAACCGA

        550        560        570        580        590        600        610        620        630
CTACTTTGGG TGTCCGTGTT TCCTTTTATT TTATTGTGGC TGCTTATGGT GACAATCACA GATTGTTATC ATAAAGCGAA TTGGATTGGC

        640        650        660        670        680        690        700        710        720
CATCCGGTGA AAGTGAGACT CATTATCTAT CTGTTTGCTG GATCCGCTCC ATTGAGTGTG TTTACTCTAA GTACAATTTC AACAGTTATT

       730        740   VP4  751                                          781                                        811
TCAATCAGAC AATTGTATCA TA ATG GGT GCT CAG GTT TCA TCA CAG AAA GTG GGC GCA CAT GAA AAC TCA AAT AGA GCG TAT GGT GGT TCT
                         MET GLY ALA GLN VAL SER SER GLN LYS VAL GLY ALA HIS GLU ASN SER ASN ARG ALA TYR GLY GLY SER

                            841                                         871                                         901
ACC ATT AAT TAC ACC ACC ATT AAT TAT TAT AGA GAT TCA GCT AGT AAC GCG GCT TCG AAA CAG GAC TTC TCT CAA GAC CCT TCC AAG TTC
THR ILE ASN TYR THR THR ILE ASN TYR TYR ARG ASP SER ALA SER ASN ALA ALA SER LYS GLN ASP PHE SER GLN ASP PRO SER LYS PHE

                            931                             ▷VP2    961                                         991
ACC GAG CCC ATC AAG GAT GTC CTG ATA AAA ACA GCC CCA ATG CTA AAC|TCG CCA AAC ATA GAG GCT TGC GGG TAT AGC GAT AGA GTA CTG
THR GLU PRO ILE LYS ASP VAL LEU ILE LYS THR ALA PRO MET LEU ASN|SER PRO ASN ILE GLU ALA CYS GLY TYR SER ASP ARG VAL LEU
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                                1021                                1051                                1081
CAA TTA ACA CTG GGA AAC TCC ACT ATA ACC ACA CAG GAG GCG GCT AAT TCA GTA GTC GCT TAT GGG CGT TGG CCT GAA TAT CTG AGG GAC
GLN LEU THR LEU GLY ASN SER THR ILE THR THR GLN GLU ALA ALA ASN SER VAL VAL ALA TYR GLY ARG TRP PRO GLU TYR LEU ARG ASP

                                1111                                1141                                1171
AGC GAA GCC AAT CCA GTG GAC CAG CCG ACA GAA CCA GAC GTC GCT GCA TGC AGG TTT TAT ACG CTA GAC ACC GTG TCT TGG ACG AAA GAG
SER GLU ALA ASN PRO VAL ASP GLN PRO THR GLU PRO ASP VAL ALA ALA CYS ARG PHE TYR THR LEU ASP THR VAL SER TRP THR LYS GLU

                                1201                                1231                                1261
TCG CGA CGG TGG TGG TGG AAG TTG CCT GAT GCA CTG AGG GAC ATG GGA CTC TTT GGG CAA AAT ATG TAC TAC CAC TAC CTA GGT AGG TCC
SER ARG GLY TRP TRP TRP LYS LEU PRO ASP ALA LEU ARG ASP MET GLY LEU PHE GLY GLN ASN MET TYR TYR HIS TYR LEU GLY ARG SER

                                1291                                1321                                1351
GGG TAC ACC GTG CAT GTA CAG TGT AAC GCC TCC AAA TTC CAC CAG GGG GCA CTA GGG GTA TTC GCC GTA CCA GAG ATG TGT CTG GCC GGG
GLY TYR THR VAL HIS VAL GLN CYS ASN ALA SER LYS PHE HIS GLN GLY ALA LEU GLY VAL PHE ALA VAL PRO GLU MET CYS LEU ALA GLY

                                1381                                1411                                1441
GAT AGC AAC ACC ACT ACC ATG CAC ACC AGC TAT CAA AAT GCC AAT CCT GGC GAG AAA CGA GGC ACT TTC ACG GGT ACG TTC ACT CCT GAC
ASP SER ASN THR THR THR MET HIS THR SER TYR GLN ASN ALA ASN PRO GLY GLU LYS GLY GLY THR PHE THR GLY THR PHE THR PRO ASP

                                1471                                1501                                1531
AAC AAC CAG ACA TCA CCT GCC CGC AGG TTC TGC CCG GTG GAT TAC CTC CTT CGA AAT GGC ACG TTG TTG GGG AAT GCC TTT GTG TTC CCG
ASN ASN GLN THR SER PRO ALA ARG ARG PHE CYS PRO VAL ASP TYR LEU LEU GLY ASN GLY THR LEU LEU GLY ASN ALA PHE VAL PHE PRO

                                1561                                1591                                1621
CAC CAG ATA ATA AAC CTA CGG ACC AAC AAC TGT GCT ACA CTG GTA CTC CCT TAC GTG AAC TCC CTC TCG ATA GAT AGT ATG GTA AAG CAC
HIS GLN ILE ILE ASN LEU ARG THR ASN ASN CYS ALA THR LEU VAL LEU PRO TYR VAL ASN SER LEU SER ILE ASP SER MET VAL LYS HIS

                                1651                                1681                                1711
AAT AAT TGG GGA ATT GCA ATA TTA CCA TTG GCC CCA TTA AAT TTT GCT AGT GAG TCG TCC CCA GAG ATT CCA ATC ACC TTG ACC ATA GCC
ASN ASN TRP GLY ILE ALA ILE LEU PRO LEU ALA PRO LEU ASN PHE ALA SER GLU SER SER PRO GLU ILE PRO ILE THR LEU THR ILE ALA

                                1741                                VP3 1771                            1801
CCT ATG TGC TGT GAG TTC AAT GGA TTA AGA AAC ATC ACC CTG CCA GGC TTA CAG GGC CTG CCG GTC ATG AAC ACC CCT GGT AGC AAT CAA
PRO MET CYS CYS GLU PHE ASN GLY LEU ARG ASN ILE THR LEU PRO ARG LEU GLN GLY LEU PRO VAL MET ASN THR PRO GLY SER ASN GLN

                                1831                                1861                                1891
TAT CTT ACT GCA GAC AAC TTC CAG TCA CCG TGT GCG CTG CCT GAA TTT GAT GTG ACC CCA CCT ATT GAC ATA CCC GGT GAA GTA AAG AAC
TYR LEU THR ALA ASP ASN PHE GLN SER PRO CYS ALA LEU PRO GLU PHE ASP VAL THR PRO PRO ILE ASP ILE PRO GLY GLU VAL LYS ASN

                                1921                                1951                                1981
ATG ATG GAA TTG GCA GAA ATC GAC ACC ATG ATT CCC TTT GAC TTA AGT GCC ACA AAA AAG AAC ACC ATG GAA ATG TAT AGG GTT CGG TTA
MET MET GLU LEU ALA GLU ILE ASP THR MET ILE PRO PHE ASP LEU SER ALA THR LYS LYS ASN THR MET GLU MET TYR ARG VAL ARG LEU

                                2011                                2041                                2071
AGT GAC AAA CCA CAT ACA GAC GAT CCC ATA CTC TGC CTG TCA CTC TCT CCA GCT TCA GAT CCT AGG TTG TCA CAT ACT ATG CTT GGA GAA
SER ASP LYS PRO HIS THR ASP ASP PRO ILE LEU CYS LEU SER LEU SER PRO ALA SER ASP PRO ARG LEU SER HIS THR MET LEU GLY GLU
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                                2101                                2131                                2161
ATC CTA AAT TAC TAC ACA CAC TGG GCA GGA TCC CTG AAG TTC ACG TTT CTG TTC TGT GGA TCC ATG ATG GCA ACT GGC AAA CTG TTG GTG
ILE LEU ASN TYR TYR THR HIS TRP ALA GLY SER LEU LYS PHE THR PHE LEU PHE CYS GLY SER MET MET ALA THR GLY LYS LEU LEU VAL

                                2191                                2221                                2251
TCA TAC GCG CCT CCT GGA GCC GAC CCA CCA AAG AAG CGT AAG GAG GCG ATG TTG GGA ACA CAT GTG ATC TGG GAC ATA GGA CTG CAG TCC
SER TYR ALA PRO PRO GLY ALA ASP PRO PRO LYS LYS ARG LYS GLU ALA MET LEU GLY THR HIS VAL ILE TRP ASP ILE GLY LEU GLN SER

                                2281                                2311                                2341
TCA TGT ACT ATG GTA GTG CCA TGG ATT AGC AAC ACC ACG TAT CGG CAA ACC ATA GAT GAT AGT TTC ACC GAA GGC GGA TAC ATC AGC GTC
SER CYS THR MET VAL VAL PRO TRP ILE SER ASN THR THR TYR ARG GLN THR ILE ASP ASP SER PHE THR GLU GLY GLY TYR ILE SER VAL

                                2371                                2401                                2431
TTC TAC CAA ACT AGA ATA GTC GTC CCT CTT TCG ACA CCC AGA GAG ATG GAC ATC CTT GGT TTT GTG TCA GCG TGT AAT GAC TTC AGC GTG
PHE TYR GLN THR ARG ILE VAL VAL PRO LEU SER THR PRO ARG GLU MET ASP ILE LEU GLY PHE VAL SER ALA CYS ASN ASP PHE SER VAL

                                2461                                VP1 2491                                2521
CGC TTG TTG CGA GAT ACC ACA CAT ATA GAG CAA AAA GCG CTA GCA CAG GGG TTA GGT CAG ATG CTT GAA AGC ATG ATT GAC AAC ACA GTC
ARG LEU LEU ARG ASP THR THR HIS ILE GLU GLN LYS ALA LEU ALA GLN GLY LEU GLY GLN MET LEU GLU SER MET ILE ASP ASN THR VAL

                                2551                                2581                                2611
CGT GAA ACG GTG GGG GCG GCA ACA TCT AGA GAC GCT CTC CCA AAC ACT GAA GCC AGT GGA CCA ACA CAC TCC AAG GAA ATT CCG GCA CTC
ARG GLU THR VAL GLY ALA ALA THR SER ARG ASP ALA LEU PRO ASN THR GLU ALA SER GLY PRO THR HIS SER LYS GLU ILE PRO ALA LEU

                                2641                                2671                                2701
ACC GCA GTG GAA ACT GGG GCC ACA AAT CCA CTA GTC CCT TCT GAT ACA GTG CAA ACC AGA CAT GTT GTA CAA CAT AGG TCA AGG TCA GAG
THR ALA VAL GLU THR GLY ALA THR ASN PRO LEU VAL PRO SER ASP THR VAL GLN THR ARG HIS VAL VAL GLN HIS ARG SER ARG SER GLU

                                2731                                2761                                2791
TCT AGC ATA GAG TCT TTC TTC GCG CGG GGT GCA TGC GTG ACC ATT ATG ACC GTG GAT AAC CCA GCT TCC ACC ACG AAT AAG GAT AAG CTA
SER SER ILE GLU SER PHE PHE ALA ARG GLY ALA CYS VAL THR ILE MET THR VAL ASP ASN PRO ALA SER THR THR ASN LYS ASP LYS LEU

                                2821                                2851                                2881
TTT GCA GTG TGG AAG ATC ACT TAT AAA GAT ACT GTC CAG TTA CGG AGG AAA TTG GAG TTC TTC ACC TAT TCT AGA TTT GAT ATG GAA CTT
PHE ALA VAL TRP LYS ILE THR TYR LYS ASP THR VAL GLN LEU ARG ARG LYS LEU GLU PHE PHE THR TYR SER ARG PHE ASP MET GLU LEU

                                2911                                2941                                2971
ACC TTT GTG GTT ACT GCA AAT TTC ACT GAG ACT AAC AAT GGG CAT GCC TTA AAT CAA GTG TAC CAA ATT ATG TAC GTA CCA CCA GGC GCT
THR PHE VAL VAL THR ALA ASN PHE THR GLU THR ASN ASN GLY HIS ALA LEU ASN GLN VAL TYR GLN ILE MET TYR VAL PRO PRO GLY ALA

                                3001                                3031                                3061
CCA GTG CCC GAG AAA TGG GAC GAC TAC ACA TGG CAA ACC TCA TCA AAT CCA TCA ATC TTT TAC ACC TAC GGA ACA GCT CCA GCC CGG ATC
PRO VAL PRO GLU LYS TRP ASP ASP TYR THR TRP GLN THR SER SER ASN PRO SER ILE PHE TYR THR TYR GLY THR ALA PRO ALA ARG ILE

                                3091                                3121                                3151
TCG GTA CCG TAT GTT GGT ATT TCG AAC GCC TAT TCA CAC TTT TAC GAC GGT TTT TCC AAA GTA CCA CTG AAG GAC CAG TCG GCA GCA CTA
SER VAL PRO TYR VAL GLY ILE SER ASN ALA TYR SER HIS PHE TYR ASP GLY PHE SER LYS VAL PRO LEU LYS ASP GLN SER ALA ALA LEU
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                                3181                                3211                                3241
GGT GAC TCC CTT TAT GGT GCA GCA TCT CTA AAT GAC TTC GGT ATT TTG GCT GTT AGA GTA GTC AAT GAT CAC AAC CCG ACC AAG GTC ACC
GLY ASP SER LEU TYR GLY ALA ALA SER LEU ASN ASP PHE GLY ILE LEU ALA VAL ARG VAL VAL ASN ASP HIS ASN PRO THR LYS VAL THR

                                3271                                3301                                3331
TCC AAA ATC AGA GTG TAT CTA AAA CCC AAA CAC ATC AGA GTC TGG TGC CCG CGT CCA CCG AGG GCA GTG GCG TAC TAC GGC CCT GGA GTG
SER LYS ILE ARG VAL TYR LEU LYS PRO LYS HIS ILE ARG VAL TRP CYS PRO ARG PRO PRO ARG ALA VAL ALA TYR TYR GLY PRO GLY VAL

                                3361                          3b
                                                             >3391                               3421
GAT TAC AAG GAT GGT ACG CTT ACA CCC CTC TCC ACC AAG GAT CTG ACC ACA TAT|GGA TTC GGA CAC CAA AAC AAA GCG GTG TAC ACT GCA
ASP TYR LYS ASP GLY THR LEU THR PRO LEU SER THR LYS ASP LEU THR THR TYR|GLY PHE GLY HIS GLN ASN LYS ALA VAL TYR THR ALA

                                3451                                3481                                3511
GGT TAC AAA ATT TGC AAC TAC CAC TTG GCC ACT CAG GAT GAT TTG CAA AAC GCA GTG AAC GTC ATG TGG AGT AGA GAC CTC TTA GTC ACA
GLY TYR LYS ILE CYS ASN TYR HIS LEU ALA THR GLN ASP ASP LEU GLN ASN ALA VAL ASN VAL MET TRP SER ARG ASP LEU LEU VAL THR

                                3541                                3571                                3601
GAA TCA AGA GCC CAG GGC ACC GAT TCA ATC GCA AGG TGC AAT TGC AAC GCA GGG GTG TAC TAC TGC GAG TCT AGA AGG AAA TAC TAC CCA
GLU SER ARG ALA GLN GLY THR ASP SER ILE ALA ARG CYS ASN CYS ASN ALA GLY VAL TYR TYR CYS GLU SER ARG ARG LYS TYR TYR PRO

                                3631                                3661                                3691
GTA TCC TTC GTT GGC CCA ACG TTC CAG TAC ATG GAG GCT AAT AAC TAT TAC CCA GCT AGG TAC CAG TCC CAT ATG CTC ATT GGC CAT GGA
VAL SER PHE VAL GLY PRO THR PHE GLN TYR MET GLU ALA ASN ASN TYR TYR PRO ALA ARG TYR GLN SER HIS MET LEU ILE GLY HIS GLY

                                3721                                3751                                3781
TTC GCA TCT CCA GGG GAT TGT GGT GGC ATA CTC AGA TGT CAC CAC GGG GTG ATA GGG ATC ATT ACT GCT GGT GGC GAA GGG TTG GTT GCA
PHE ALA SER PRO GLY ASP CYS GLY GLY ILE LEU ARG CYS HIS HIS GLY VAL ILE GLY ILE ILE THR ALA GLY GLY GLU GLY LEU VAL ALA

                                3811                          5b                                                    3871
TTT TCA GAC ATT AGA GAC TTG TAT GCC TAC GAA GAA GAA GCC ATG GAA CAA|GGC ATC ACC AAT TAC ATA GAG TCA CTT GGG GCC GCA TTT
PHE SER ASP ILE ARG ASP LEU TYR ALA TYR GLU GLU GLU ALA MET GLU GLN|GLY ILE THR ASN TYR ILE GLU SER LEU GLY ALA ALA PHE

                                3901                                3931                                3961
GGA AGT GGA TTT ACT CAG CAG ATT AGC GAC AAA ATA ACA GAG TTG ACC AAT ATG GTG ACC AGT ACC ATC ACT GAA AAG CTA CTT AAG AAC
GLY SER GLY PHE THR GLN GLN ILE SER ASP LYS ILE THR GLU LEU THR ASN MET VAL THR SER THR ILE THR GLU LYS LEU LEU LYS ASN

                                3991                                4021                                4051
TTG ATC AAG ATC ATA TCC TCA CTA GTT ATT ATA ACT AGG AAC TAT GAA GAC ACC ACA ACA GTG CTC GCT ACC CTG GCC CTT CTT GGG TGT
LEU ILE LYS ILE ILE SER SER LEU VAL ILE ILE THR ARG ASN TYR GLU ASP THR THR THR VAL LEU ALA THR LEU ALA LEU LEU GLY CYS

                                4081                                4111                                X   4141
GAT GCT TCA CCA TGG CAG TGG CTT AGA AAG AAA GCA TGC GAT GTT CTG GAG ATA CCT TAT GTC ATC AAG CAA|GGT GAC AGT TGG TTG AAG
ASP ALA SER PRO TRP GLN TRP LEU ARG LYS LYS ALA CYS ASP VAL LEU GLU ILE PRO TYR VAL ILE LYS GLN|GLY ASP SER TRP LEU LYS

                                4171                                4201                                4231
AAG TTT ACT GAA GCA TGC AAC GCA GCT AAG GGA CTG GAG TGG GTG TCA AAC AAA ATC TCA AAA TTC ATT GAT TGG CTC AAG GAG AAA ATT
LYS PHE THR GLU ALA CYS ASN ALA ALA LYS GLY LEU GLU TRP VAL SER ASN LYS ILE SER LYS PHE ILE ASP TRP LEU LYS GLU LYS ILE
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                                    4261                                    4291                                    4321
ATC CCA CAA GCT AGA GAT AAG TTG GAA TTT GTA ACA AAA CTT AGA CAA CTA GAA ATG CTG GAA AAC CAA ATC TCA ACT ATA CAC CAA TCA
ILE PRO GLN ALA ARG ASP LYS LEU GLU PHE VAL THR LYS LEU ARG GLN LEU GLU MET LEU GLU ASN GLN ILE SER THR ILE HIS GLN SER

                                    4351                                    4381                                    4411
TGC CCT AGT CAG GAA CAC CAG GAA ATT CTA TTC AAT AAT GTC AGA TGG TTA TCC ATC CAG TCT AAG AGG TTT GCC CCT GTT TAC GCA GTG
CYS PRO SER GLN GLU HIS GLN GLU ILE LEU PHE ASN ASN VAL ARG TRP LEU SER ILE GLN SER LYS ARG PHE ALA PRO LEU TYR ALA VAL

                                    4441                                    4471                                    4501
GAA GCC AAA AGA ATA CAG AAA CTA GAG CAT ACT ATT AAC AAC TAC ATA CAG TTC AAG AGC AAA CAC CGT ATT GAA CCA GTA TGT TTG CTA
GLU ALA LYS ARG ILE GLN LYS LEU GLU HIS THR ILE ASN ASN TYR ILE GLN PHE LYS SER LYS HIS ARG ILE GLU PRO VAL CYS LEU LEU

                                    4531                                    4561                                    4591
GTA CAT GGC AGC CCC GGA ACA GGT AAA TCT GTA GCA ACC AAC CTG ATT GCT AGA GCC ATA GCT GAA AGA GAA AAC ACG TCC ACG TAC TCG
VAL HIS GLY SER PRO GLY THR GLY LYS SER VAL ALA THR ASN LEU ILE ALA ARG ALA ILE ALA GLU ARG GLU ASN THR SER THR TYR SER

                                    4621                                    4651                                    4681
CTA CCC CCG GAT CCA TCA CAC TTC GAC GGA TAC AAA CAA CAG GGA GTG GTG ATT ATG GAC GAC CTG AAT CAA AAC CCA GAT GGT GCG GAC
LEU PRO PRO ASP PRO SER HIS PHE ASP GLY TYR LYS GLN GLN GLY VAL VAL ILE MET ASP ASP LEU ASN GLN ASN PRO ASP GLY ALA ASP

                                    4711                                    4741                                    4771
ATG AAG CTG TTC TGT CAG ATG GTA TCA ACA GTG GAG TTT ATA CCA CCC ATG GCA TCC CTG GAG GAG AAA GGA ATC CTG TTT ACT TCA AAT
MET LYS LEU PHE CYS GLN MET VAL SER THR VAL GLU PHE ILE PRO PRO MET ALA SER LEU GLU GLU LYS GLY ILE LEU PHE THR SER ASN

                                    4801                                    4831                                    4861
TAC GTT CTA GCA TCC ACA AAC TCA AGC AGA ATT TCC CCC CCC ACT GTG GCA CAC AGT GAT GCA TTA GCC AGG CGC TTT GCG TTC GAC ATG
TYR VAL LEU ALA SER THR ASN SER SER ARG ILE SER PRO PRO THR VAL ALA HIS SER ASP ALA LEU ALA ARG ARG PHE ALA PHE ASP MET

                                    4891                                    4921                                    4951
GAC ATT CAG GTC ATG AAT GAG TAT TCT AGA GAT GGG AAA TTG AAC ATG GCC ATG GCT ACT GAA ATG TGT AAG AAC TGT CAC CAA CCA GCA
ASP ILE GLN VAL MET ASN GLU TYR SER ARG ASP GLY LYS LEU ASN MET ALA MET ALA THR GLU MET CYS LYS ASN CYS HIS GLN PRO ALA

                                    4981                                    5011                                    5041
AAC TTT AAG AGA TGC TGT CCT TTA GTG TGT GGT AAG GCA ATT CAA TTA ATG GAC AAA TCT TCC AGA GTT AGA TAC AGT ATT GAC CAG ATC
ASN PHE LYS ARG CYS CYS PRO LEU VAL CYS GLY LYS ALA ILE GLN LEU MET ASP LYS SER SER ARG VAL ARG TYR SER ILE ASP GLN ILE

                                    5071                                    5101                    1b              5131
ACT ACA ATG ATT ATC AAT GAG AGA AAC AGA AGA TCC AAC ATT GGC AAT TGT ATG GAG GCT TTG TTT CAA|GGA CCA CTC CAG TAT AAA GAC
THR THR MET ILE ILE ASN GLU ARG ASN ARG ARG SER ASN ILE GLY ASN CYS MET GLU ALA LEU PHE GLN|GLY PRO LEU GLN TYR LYS ASP

                                    5161                                    5191                                    5221
TTG AAA ATT GAC ATC AAG ACG AGT CCC CCT GCT GAA TGT ATC AAT GAC TTG CTC CAA GCA GTT GAC TCC CAG GAG GTG AGA GAT TAC TGT
LEU LYS ILE ASP ILE LYS THR SER PRO PRO PRO GLU CYS ILE ASN ASP LEU LEU GLN ALA VAL ASP SER GLN GLU VAL ARG ASP TYR CYS

                                    5251                                    5281                                    5311
GAG AAG AAG GGT TGG ATA GTC AAC ATC ACC AGC CAG GTT CAA ACA GAA AGG AAC ATC AAC AGG GCA ATG ACA ATT CTA CAA GCG GTG ACA
GLU LYS LYS GLY TRP ILE VAL ASN ILE THR SER GLN VAL GLN THR GLU ARG ASN ILE ASN ARG ALA MET THR ILE LEU GLN ALA VAL THR
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                5341                                    5371 ▷VPg                       5401
ACC TTC GCC GCA GTG GCT GGA GTT GTC TAT GTC ATG TAT AAA CTG TTT GCT GGA CAC CAG|GGA GCA TAC ACT GGT TTA CCA AAC AAA AAA
THR PHE ALA ALA VAL ALA GLY VAL VAL TYR VAL MET TYR LYS LEU PHE ALA GLY HIS GLN|GLY ALA TYR THR GLY LEU PRO ASN LYS LYS

                5431         ▷2                         5461                            5491
CCC AAC GTG CCC ACC ATT CGG ACA GCA AAG GTA CAA|GGA CCA GGG TTC GAT TAC GCA GTG GCT ATG GCT AAA AGA AAC ATT GTT ACA GCA
PRO ASN VAL PRO THR ILE ARG THR ALA LYS VAL GLN|GLY PRO GLY PHE ASP TYR ALA VAL ALA MET ALA LYS ARG ASN ILE VAL THR ALA

                5521                                    5551                            5581
ACT ACT AGC AAG GGA GAG TTC ACT ATG TTA GGA GTC CAC GAC AAC GTG GCT ATT TTA CCA ACC CAC GCT TCA CCT GGT GAA AGC ATT GTG
THR THR SER LYS GLY GLU PHE THR MET LEU GLY VAL HIS ASP ASN VAL ALA ILE LEU PRO THR HIS ALA SER PRO GLY GLU SER ILE VAL

                5611                                    5641                            5671
ATC GAT GGC AAA GAA GTG GAG ATC TTG GAT GCC AAA GCG CTC GAA GAT CAA GCA GGA ACC AAT CTT GAA ATC ACT ATA ATC ACT CTA AAG
ILE ASP GLY LYS GLU VAL GLU ILE LEU ASP ALA LYS ALA LEU GLU ASP GLN ALA GLY THR ASN LEU GLU ILE THR ILE ILE THR LEU LYS

                5701                                    5731                            5761
AGA AAT GAA AAG TTC AGA GAC ATT AGA CCA CAT ATA CCT ACT CAA ATC ACT GAG ACA AAT GAT GGA GTC TTG ATC GTG AAC ACT AGC AAG
ARG ASN GLU LYS PHE ARG ASP ILE ARG PRO HIS ILE PRO THR GLN ILE THR GLU THR ASN ASP GLY VAL LEU ILE VAL ASN THR SER LYS

                5791                                    5821                            5851
TAC CCC AAT ATG TAT GTT CCT GTC GGT GCT GTG ACT GAA CAG GGA TAT CTA AAT CTC GGT GGG CGC CAA ACT GCT CGT ACT CTA ATG TAC
TYR PRO ASN MET TYR VAL PRO VAL GLY ALA VAL THR GLU GLN GLY TYR LEU ASN LEU GLY GLY ARG GLN THR ALA ARG THR LEU MET TYR

                5881                                    5911                            5941
AAC TTT CCA ACC AGA GCA GGA CAG TGT GGT GGA GTC ATC ACA TGT ACT GGG AAA GTC ATC GGG ATG CAT GTT GGT GGG AAC GGT TCA CAC
ASN PHE PRO THR ARG ALA GLY GLN CYS GLY GLY VAL ILE THR CYS THR GLY LYS VAL ILE GLY MET HIS VAL GLY GLY ASN GLY SER HIS

                5971                       ▷4 (p63)     6001                            6031
GGG TTT GCA GCG GCC CTG AAG CGA TCA TAC TTC ACT CAG AGT CAA|GGT GAA ATC CAG TGG ATG AGA CCT TCG AAG GAA GTG GGA TAT CCA
GLY PHE ALA ALA ALA LEU LYS ARG SER TYR PHE THR GLN SER GLN|GLY GLU ILE GLN TRP MET ARG PRO SER LYS GLU VAL GLY TYR PRO

                6061                                    6091                            6121
ATC ATA AAT GCC CCG TCC AAA ACC AAG CTT GAA CCC AGT GCT TTC CAC TAT GTG TTT GAA GGG GTG AAG GAA CCA GCA GTC CTC ACT AAA
ILE ILE ASN ALA PRO SER LYS THR LYS LEU GLU PRO SER ALA PHE HIS TYR VAL PHE GLU GLY VAL LYS GLU PRO ALA VAL LEU THR LYS

                6151                                    6181                            6211
AAC GAT CCC AGG CTT AAG ACA GAC TTT GAG GAG GCA ATT TTC TCC AAG TAC GTG GGT AAC AAA ATT ACT GAA GTG GAT GAG TAC ATG AAA
ASN ASP PRO ARG LEU LYS THR ASP PHE GLU GLU ALA ILE PHE SER LYS TYR VAL GLY ASN LYS ILE THR GLU VAL ASP GLU TYR MET LYS

                6241                                    6271                            6301
GAG GCA GTA GAC CAC TAT GCT GGC CAG CTC ATG TCA CTA GAC ATC AAC ACA GAA CAA ATG TGC TTG GAG GAT GCC ATG TAT GGC ACT GAT
GLU ALA VAL ASP HIS TYR ALA GLY GLN LEU MET SER LEU ASP ILE ASN THR GLU GLN MET CYS LEU GLU ASP ALA MET TYR GLY THR ASP
------------------------------------------------------------------------------------------------------------------------
                6331                                    6361                            6391
GGT CTA GAA GCA CTT GAT TTG TCC ACC AGT GCT GGC TAC CCT TAT GTA GCA ATG GGA AAG AAG AAG AGA GAC ATC TTG AAC AAA CAA ACC
GLY LEU GLU ALA LEU ASP LEU SER THR SER ALA GLY TYR PRO TYR VAL ALA MET GLY LYS LYS LYS ARG ASP ILE LEU ASN LYS GLN THR
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                                    6421                                    6451                                    6481
AGA GAC ACT AAG GAA ATG CAA AAA CTG CTC GAC ACA TAT GGA ATC AAC CTC CCA CTG GTG ACT TAT GTA AAG GAT GAA CTT AGA TCC AAA
ARG ASP THR LYS GLU MET GLN LYS LEU LEU ASP THR TYR GLY ILE ASN LEU PRO LEU VAL THR TYR VAL LYS ASP GLU LEU ARG SER LYS

                                    6511                                    6541                                    6571
ACA AAG GTT GAG CAG GGG AAA TCC AGA TTA ATT GAA GCT TCT AGT TTG AAT GAC TCA GTG GCA ATG AGA ATG GCT TTT GGG AAC CTA TAT
THR LYS VAL GLU GLN GLY LYS SER ARG LEU ILE GLU ALA SER SER LEU ASN ASP SER VAL ALA MET ARG MET ALA PHE GLY ASN LEU TYR

                                    6601                                    6631                                    6661
GCT GCT TTT CAC AAA AAC CCA GGA GTG ATA ACA GGT TCA GCA GTG GGG TGC GAT CCA GAT TTG TTT TGG AGC AAA ATT CCG GTA TTG ATG
ALA ALA PHE HIS LYS ASN PRO GLY VAL ILE THR GLY SER ALA VAL GLY CYS ASP PRO ASP LEU PHE TRP SER LYS ILE PRO VAL LEU MET

                                    6691                                    6721                                    6751
GAA GAG AAG CTG TTT GCT TTT GAC TAC ACA GGG TAT GAT GCA TCT CTC AGC CCT GCT TGG TTC GAG GCA CTA AAG ATG GTG CTT GAG AAA
GLU GLU LYS LEU PHE ALA PHE ASP TYR THR GLY TYR ASP ALA SER LEU SER PRO ALA TRP PHE GLU ALA LEU LYS MET VAL LEU GLU LYS

                                    6781                                    6811                                    6841
ATC GGA TTC GGA GAC AGA GTT GAC TAC ATC GAC TAC CTA AAC CAC TCA CAC CAC CTG TAC AAG AAT AAA ACA TAC TGT GTC AAG GGC GGT
ILE GLY PHE GLY ASP ARG VAL ASP TYR ILE ASP TYR LEU ASN HIS SER HIS HIS LEU TYR LYS ASN LYS THR TYR CYS VAL LYS GLY GLY

                                    6871                                    6901                                    6931
ATG CCA TCT GGC TGC TCA GGC ACT TCA ATT TTT AAC TCA ATG ATT AAC AAC TTG ATT ATC AGG ACA CTC TTA CTG AAA ACC TAC AAG GGC
MET PRO SER GLY CYS SER GLY THR SER ILE PHE ASN SER MET ILE ASN ASN LEU ILE ILE ARG THR LEU LEU LEU LYS THR TYR LYS GLY

                                    6961                                    6991                                    7021
ATA GAT TTA GAC CAC CTA AAA ATG ATT GCC TAT GGT GAT GAT GTA ATT GCT TCC TAC CCC CAT GAA GTT GAC GCT AGT CTC CTA GCC CAA
ILE ASP LEU ASP HIS LEU LYS MET ILE ALA TYR GLY ASP ASP VAL ILE ALA SER TYR PRO HIS GLU VAL ASP ALA SER LEU LEU ALA GLN

                                    7051                                    7081                                    7111
TCA GGA AAA GAC TAT GGA CTA ACT ATG ACT CCA GCT GAC AAA TCA GCT ACA TTT GAA ACA GTC ACA TGG GAG AAT GTA ACA TTC TTG AAG
SER GLY LYS ASP TYR GLY LEU THR MET THR PRO ALA ASP LYS SER ALA THR PHE GLU THR VAL THR TRP GLU ASN VAL THR PHE LEU LYS

                                    7141                                    7171                                    7201
AGA TTC TTC AGG GCA GAC GAG AAA TAC CCA TTT CTT ATT CAT CCA GTA ATG CCA ATG AAG GAA ATT CAT GAA TCA ATT AGA TGG ACT AAA
ARG PHE PHE ARG ALA ASP GLU LYS TYR PRO PHE LEU ILE HIS PRO VAL MET PRO MET LYS GLU ILE HIS GLU SER ILE ARG TRP THR LYS

                                    7231                                    7261                                    7291
GAT CCT AGG AAC ACT CAG GAT CAC GTT CGC TCT CTG TGC CTT TTA GCT TGG CAC AAT GGC GAA GAA GAA TAT AAC AAA TTC CTA GCT AAA
ASP PRO ARG ASN THR GLN ASP HIS VAL ARG SER LEU CYS LEU LEU ALA TRP HIS ASN GLY GLU GLU GLU TYR ASN LYS PHE LEU ALA LYS

                                    7321                                    7351                                    7385
ATC AGG AGT GTG CCA ATT GGA AGA GCT TTA TTG CTC CCA GAG TAC TCA ACA TTG TAC CGC CGT TGG CTT GAC TCA TTT TAG TAA GCCTAGCTCA
ILE ARG SER VAL PRO ILE GLY ARG ALA LEU LEU LEU PRO GLU TYR SER THR LEU TYR ARG ARG TRP LEU ASP SER PHE END END

    7395        7405        7415        7425        7435
GTCGAATTGG ATTGGGTCAT ACTGTTGTAG GGGTAAATTT TTCTTTAATT CGGAG —poly(A)
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EXAMPLE 6

Transfection of Cells With
Full-Length Poliovirus Clone

CV-1 and HeLa cells were grown to 80% confluence in 10-cm plastic dishes. Briefly, the cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% calf serum. Cells were maintained at 37°C in a humidified incubator containing an atmosphere supplemented with 5% CO_2.

Cells were transfected with either 10 µg plasmid DNA per dish or 2 µg of viral RNA prepared as described in Example 1. Transfection was achieved using the modified calcium-phosphate technique described by Parker and Stark. See B. A. Parker and G. R. Stark, J. Virol., 31, 360 (1979). Briefly, medium was removed from the cells, and DNA was added as a calcium-phosphate precipitate in Hepes-buffered saline. After 20 minutes at room temperature, cells were covered with warm medium (Dulbecco's modified Eagle medium plus 10% calf serum) and incubated four hours at 37°. After this time, medium was removed, cells were washed once with warm medium, and 2.5 ml per dish of 15% glycerol in Hepes-buffered saline was added. After 3.5 minutes at 37°C, the glycerol was removed and cells were washed once with warm medium. One of the duplicate dishes was then covered with warm medium, and the other was covered with medium containing 1% agarose (Sigma). Plates were incubated at 37°C for 4-5 days. To count plaques, the agar overlay was removed and cells were stained with 0.1% crystal violet in 50% ethanol. Medium from cells incubated under liquid was assayed for infectious poliovirus on HeLa cell monolayers.

The results are shown in Table 2, wherein virus titers shown are values for typical experiments.

For RNAse treatment, 5 µg of boiled pancreatic RNAse (Worthington) were used for 10 µg DNA or 2 µg RNA.

As can be seen, a high virus titer was found in the medium from cells transfected with pVR106 but no virus was released from pBR322-transfected cells. Cells transfected with pVR106 and incubated under agar displayed, upon staining with crystal violet, plaques similar to those induced by poliovirus. No plaques were observed on cells transfected with pBR322 DNA. Usually, from 10-70 plaques were observed per 100 mm plate of cells transfected with 10 µg of pVR106. If approximately 10% of the cells receive DNA, as suggested by Parker and Stark, then infectious foci arose at an efficiency of about $2\text{-}8 \times 10^{-5}$.

Transfection with an independently-derived, full-length poliovirus cDNA clone, designated pVR106a, (see Example 4), also yielded infectious virus following transfection into cells.

Further experiments, the results of which are also presented in Table 2, illustrated that virus production in transfected cells was directed by plasmid pVR106. No virus was detected in cells transfected with pVR106 DNA cleaved by Hinf I, an enzyme which cuts the plasmid at 45 sites and which does not reduce the infectivity of viral RNA. The infectivity of pVR106 DNA was not significantly reduced by treatment with RNAse under conditions which abolish the infectivity of viral RNA. Therefore, infectivity was not due to viral RNA contaminating the pVR106 DNA. Phenol extraction of pVR106 DNA alone or followed by RNAse treatment did not lower the infectivity of the plasmid. Thus, virions did not appear

to be present in the pVR106 preparation. When pVR106 DNA was assayed directly for contaminating virions on HeLa cell monolayers, no infectivity was detected (data not shown). These results indicate that the infectivity of pVR106 is inherent in the plasmid DNA.

TABLE 2

Transfection of Cultured Mammalian Cells With Various Plasmid DNA's

Nucleic Acid	Plaque forming units/ml in medium	No. of plaques on transfected cell monolayers
CV-1 cells		
pVR106	1.2×10^9	22
pVR106 + Hinf I	0	0
pVR106 + RNAse	1.3×10^9	10
pVR106, phenol extracted	1×10^9	22
pVR106, phenol extracted, then RNAse	1.4×10^9	26
pBR322	0	0
viral RNA	1.5×10^9	71
viral RNA + RNAse	0	0
viral RNA + Hinf I	1.4×10^9	20
HeLa cells		
pVR106	3.7×10^8	69
pBR322	0	0

EXAMPLE 7

Antibody Neutralization Test

The identity of the virus produced in pVR106-transfected cells was examined employing an antibody neutralization test. About 100 plaque-forming units of the virus released into the culture medium of pVR106 transfected cells obtained following the procedures of Example 6 was mixed with various dilutions of rabbit anti-poliovirus antiserum and assayed for infectivity. In parallel, 100 plaque-forming units of authentic poliovirus was assayed for neutralization by the serum. Both viruses were neutralized 50% by a 1/50,000 dilution of the serum indicating that the pVR106-derived virus was authentic poliovirus.

EXAMPLE 8

Transfection of Cells With Poliovirus clone pVR104

Plasmid pVR104, prepared as described in Example 2 above, contains an almost full-length cDNA sequence for the entire poliovirus genome, but lacks the first 115 bases of poliovirus genome. Plasmid pVR104 was transfected into CV-1 cells following the procedures of Example 6 but did not result in virus production indicating that the 5' end of the poliovirus RNA is required for infectivity even though it does not encode protein (there are no AUG codons in this region).

Industrial Applicability

The invention described herein is useful in the production of RNA viral cDNA, such as poliovirus cDNA, by recombinant DNA techniques. The products, such as poliovirus cDNA, are in turn useful in assays for the detection of such viruses and in the production of viral antigens and antibodies against such viruses, etc.

Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

Claims

1. A method for producing RNA viral cDNA, comprising:

a. reverse transcribing RNA viral sequences to provide cDNA;

b. inserting said cDNA into a recombinant DNA vector;

c. transforming cells in which said recombinant DNA vector will multiply;

d. cloning a transformed cell to produce a clonal cell line capable of replicating said cDNA;

e. culturing said clonal cell line under conditions whereby cDNA is produced; and,

f. harvesting said human cDNA from said cell culture.

2. A method of Claim 1 wherein said RNA viral sequences comprise RNA from a human picornavirus.

3. A method of Claim 1 wherein said RNA viral sequences comprise poliovirus RNA.

4. A method of Claims 1, 2 or 3 wherein said recombinant DNA vector comprises a vector selected from a bacterial plasmid, a phage, an animal virus, or a yeast vector.

5. A method for the bacterial production of poliovirus cDNA, comprising:

a. reverse transcribing poliovirus RNA to provide ss poliovirus cDNA;

b. forming ds cDNA from said ss poliovirus cDNA;

c. inserting said ds poliovirus cDNA into a bacterial plasmid to create a chimeric plasmid;

d. transforming bacterial cells with said chimeric plasmid;

e. cloning a transformed bacterial cell to produce a clonal bacterial cell line capable of replicating said poliovirus ds cDNA;

f. culturing said clonal bacterial cell line in cell culture under conditions conducive to the production of poliovirus ds cDNA; and,

g. harvesting said poliovirus ds cDNA from said cell culture.

6. A method of Claim 5 wherein said bacterial cells comprise E. coli cells.

7. A method of Claim 6 wherein said bacterial plasmid contains a selective marker for transformed cells.

8. A.method of Claim 7 wherein said selective marker is resistant to an antibiotic.

9. A method of Claim 8 wherein said bacterial plasmic comprises plasmid pBR322.

10. Poliovirus ds cDNA produced by a method of Claims 5, 6, 7, 8 or 9.

11. A recombinant DNA vector containing RNA viral cDNA.

12. A recombinant DNA vector of Claim 11 wherein said RNA viral cDNA comprises poliovirus cDNA.

13. A chimeric bacterial plasmid containing RNA viral cDNA.

14. A chimeric bacterial plasmid of Claim 13 wherein said RNA viral cDNA comprises poliovirus ds cDNA.

15. A clonal cell line transformed with a recombinant DNA vector containing RNA viral cDNA.

16. A clonal cell line of Claim 15 wherein said RNA viral cDNA comprises poliovirus ds cDNA.

17. A clonal bacterial cell line transformed with a chimeric plasmid containing RNA viral cDNA.

18. A clonal bacterial cell line of Claim 17 wherein said RNA viral cDNA comprises poliovirus ds cDNA.

19. A clonal bacterial cell line of Claims 17 or 18 wherein said bacterial cells comprise transformed E. coli cells.

20. The clonal bacterial cell line identified by ATCC Accession No. 31884.

21. In an assay for an RNA virus, the improvement comprising employing labeled cDNA representing said RNA virus to bind to said RNA virus and thereafter detecting labeled cDNA bound to said RNA virus.

22. The improvement of Claim 21 wherein said RNA virus comprises poliovirus.

23. An assay for the detection of an RNA virus in a sample, comprising:

a. isolating an RNA-containing fraction of said sample, said RNA-containing fraction being a fraction which would contain said RNA virus if it were originally present in said sample;

b. labeling cDNA for said RNA virus;

c. incubating said labeled cDNA with said RNA-containing fraction in an incubation mixture under conditions sufficient for labeled cDNA to bind to the RNA virus;

d. removing unbound labeled cDNA from the incubation mixture; and,

e. detecting remaining bound labeled cDNA.

24. An assay of Claim 23 wherein said RNA virus comprises poliovirus.

25. An assay of Claim 24 wherein said sample comprises a human physiological sample.

26. An assay of Claim 25 wherein said human physiological sample comprises a biopsy or cerebrospinal fluid.

27. An assay of Claim 24 wherein said sample comprises sewerage.

28. A method of producing antibodies against viral RNA comprising:

a. reverse transcribing said viral RNA to provide viral RNA cDNA;

b. inserting said cDNA into a recombinant DNA vector;

c. transforming cells in which said recombinant DNA vector can multiply;

d. cloning a transformed cell to produce a clonal cell line capable of replicating said cDNA;

e. culturing said clonal cell line under conditions whereby cDNA is produced;

f. harvesting said cDNA from said cell culture;

g. selecting and isolating specific cDNA capable of directing viral RNA antigen synthesis in cells;

h. inserting said specific cDNA into cells;

i. culturing said cells under conditions whereby viral RNA antigen is produced;

j. immunizing a host with said antigen to cause said host to produce antibodies against said viral RNA.

29. A method of Claim 28 wherein said viral RNA comprises poliovirus.

30. A method of Claim 28 wherein said viral RNA comprises coxsackie virus.

31. A method of Claim 28 wherein said viral RNA comprises a rhinovirus.

32. A method of producing viral RNA antigen, comprising:

a. reverse transcribing viral RNA to provide viral RNA cDNA;

b. inserting said cDNA into a recombinant DNA vector;

c. transforming cells in which said recombinant DNA vector can multiply;

d. cloning a transformed cell to produce a clonal cell line capable of replicating said cNDA;

e. culturing said clonal cell line under conditions whereby cDNA is produced;

f. harvesting said cDNA from said cell culture;

g. selecting and isolating specific cDNA capable of directing viral RNA antigen synthesis in cells;

h. inserting said specific cDNA into cells;

i. culturing said cells under conditions whereby viral RNA antigen is produced; and,

j. harvesting said antigen.

33. Plasmid pVR106.

34. A recombinant DNA vector containing a full-length poliovirus cDNA.

35. Poliovirus cDNA having the base sequence set forth in Table I and equivalents thereto containing different codons for the same amino acid sequences or equivalent sequences.

36. Infectious RNA viral cDNA.

37. Infectious poliovirus cDNA.

38. A method for producing infectious RNA virus, comprising:

a. transfecting cells with infectious cDNA for said RNA virus;

b. culturing said cells under conditions sufficient for the cellular production of RNA virus; and,

c. harvesting said RNA virus.

39. A method of Claim 38 wherein said RNA virus is a virus having a genome comprising a single positive strand of RNA.

40. A method of Claim 38 wherein said RNA virus is poliovirus.

41. A method of Claims 38, 39 or 40 wherein said cells comprise mammalian cells.

42. A method of Claim 41 wherein said mammalian cells are human cells.

43. A method of producing vaccines from RNA viral sequences, comprising:

a. reverse transcribing said RNA viral sequences to produce infectious RNA viral cDNA;

b. mutagenizing said infectious RNA viral cDNA;

c. transfecting cells capable of viral RNA production with said mutagenized infectious RNA viral cDNA;

d. culturing said transfected cells under conditions sufficient for the production of attenuated viral RNA.

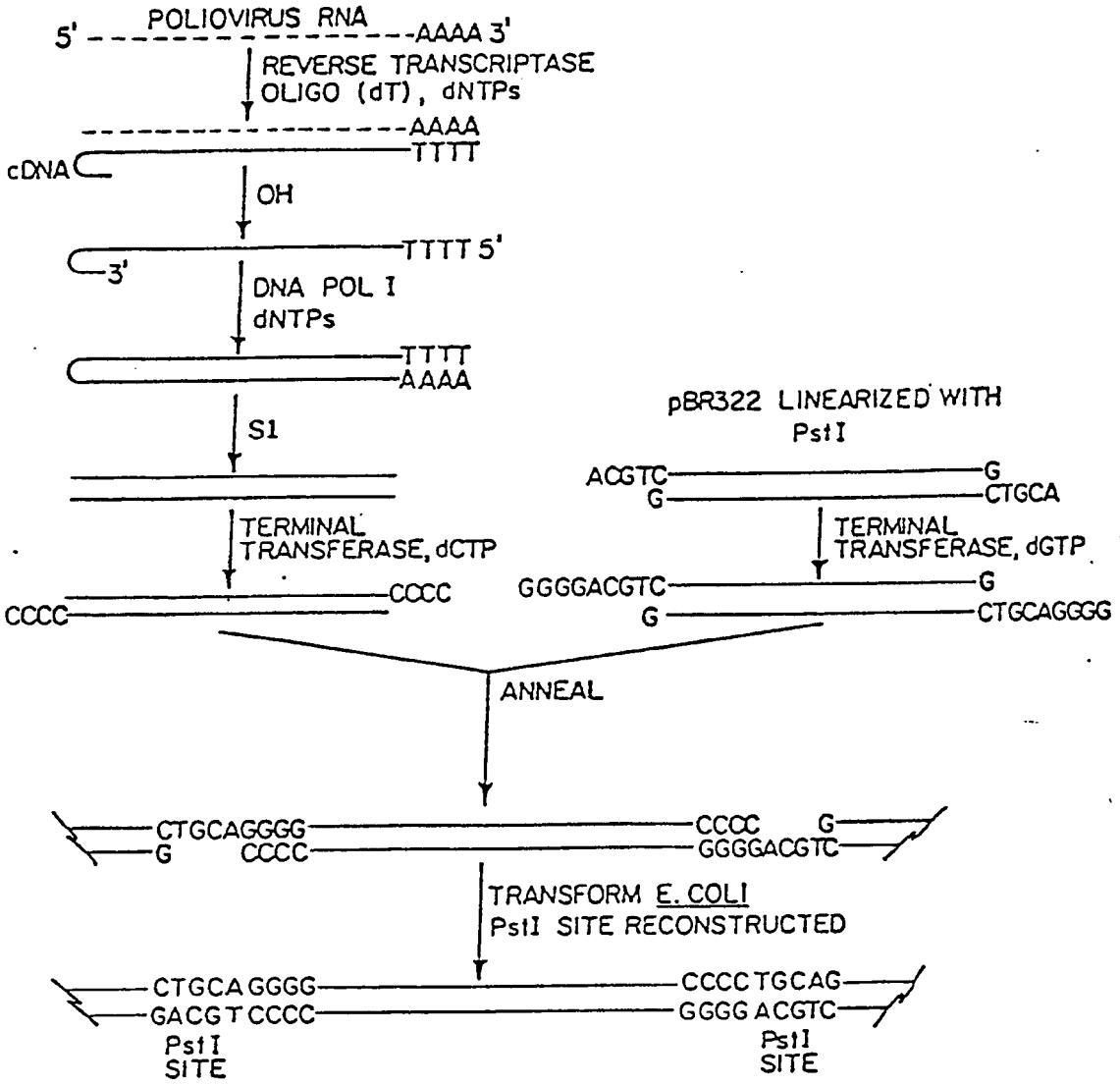

Fig. 1

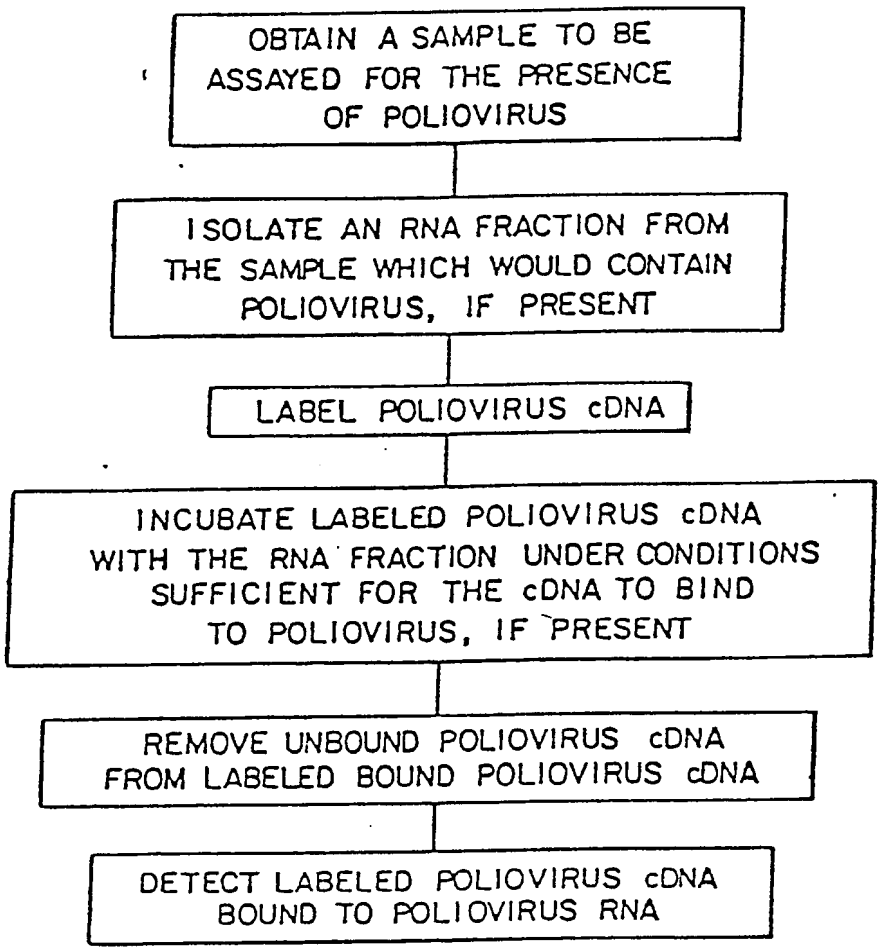

Fig. 2

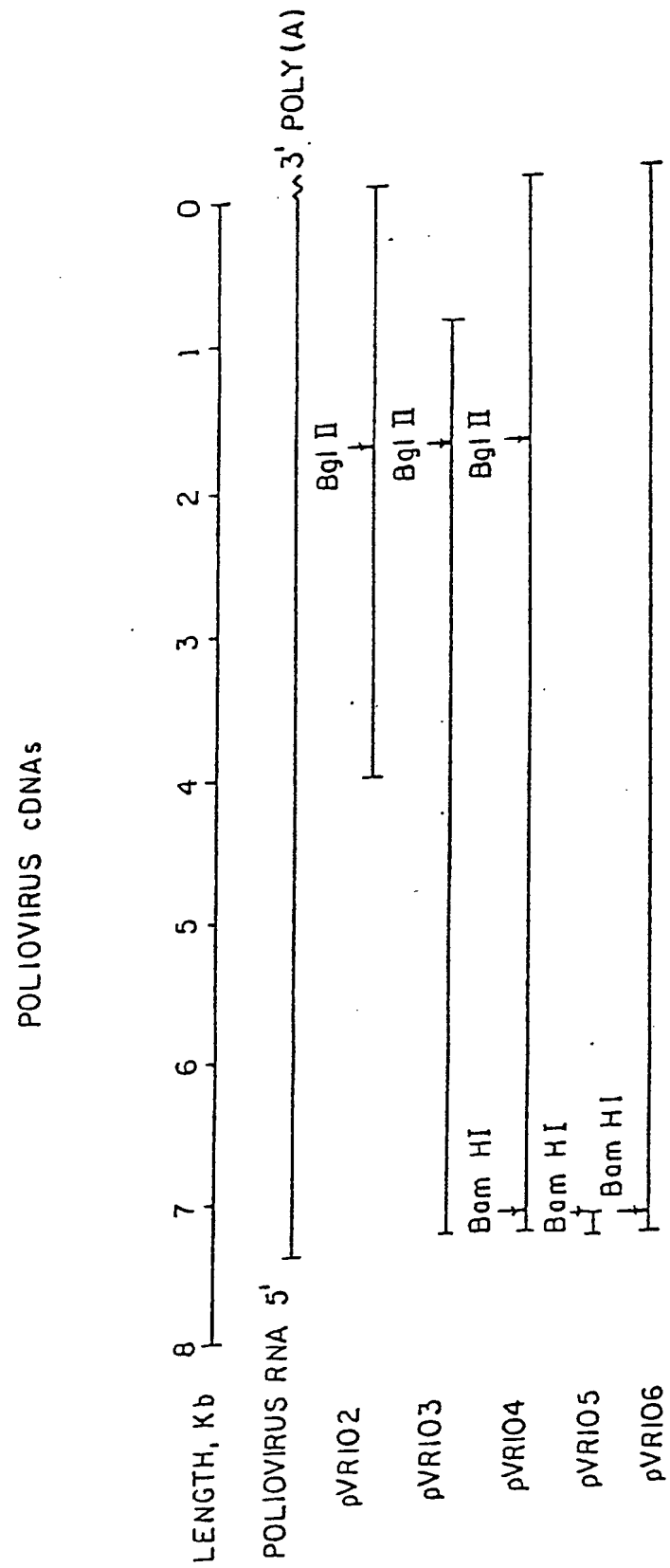

Fig. 3

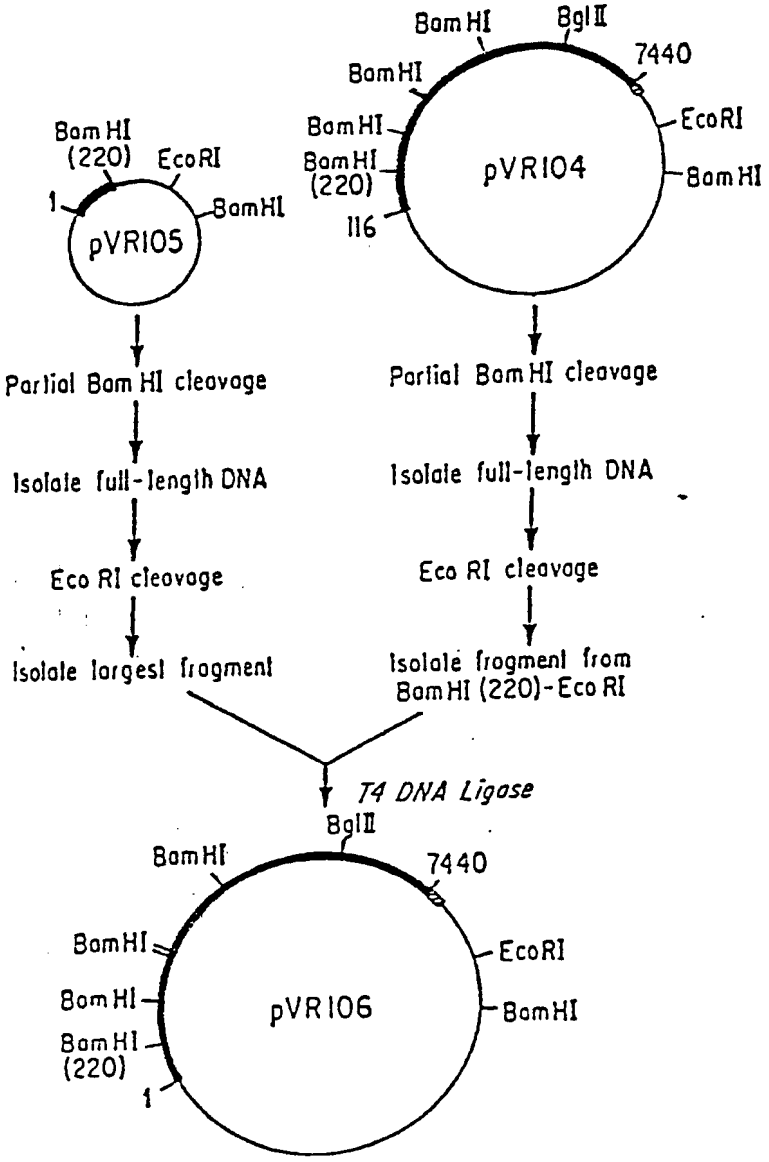

Fig. 4

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No PCT/US82/00467

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL.[3] C12N 15/00
U.S. CL. 435/172

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/172,317,235,236,239,4,5,7,89; 424/85,88,86,89

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

CHEMICAL ABSTRACTS VOLUME: 95-89

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
A	US,A, 4,237,224, PUBLISHED 02 DECEMBER 1980, COHEN.	1-43
A	DD, 0,143,794, PUBLISHED 10 SEPTEMBER 1980, AKAD WISSENSCHAFT DDR.	1-43
A	N, PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, VOL. 77, ISSUED 1980, "SEQUENCE OF 1060 3'-TERMINAL NUCLEOTIDE OF POLIOVIRUS RNA AS DETERMINED BY A MODIFICATION OF THE DIDEOXYNUCLEOTIDE METHOD" N. KITAMURA ET AL, SEE PAGES 3196-3200.	1-43
A	N, PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, VOL. 75, ISSUED 1978, "DETECTION OF VIRAL SEQUENCES OF LOW REITERATION FREQUENCY BY IN SITU HYBRIDIZATION" M. BRAHIC ET AL,SEE PAGES 6125-6129.	21-27
A,P	US,A, 4,271,145, PUBLISHED 02 JUNE 1981, WANDS.	28-31
A	N, PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, VOL. 78, ISSUED 1981, "MOLECULAR CLONING OF POLIOVIRUS cDNA AND DETERMINATION OF THE COMPLETE NUCLEOTIDE SEQUENCE OF THE VIRAL GENOME" V.R. RACANIELLO ET AL, SEE PAGES 4887-4891.	1-43

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [1]	Date of Mailing of this International Search Report [2]
29 JULY 1982	18 AUG 1982
International Searching Authority [1]	Signature of Authorized Officer [20]
RO/US	KATHLEEN S. Mc COWIN